Soy and whey hydrolysate induced cytokine expression in dendritic cells and intestinal epithelial cells

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Abstract

Hydrolyzed soy and cow's milk proteins have recently been found to possess immunomodulatory capacities. The mechanisms underlying these effects have not yet been fully elucidated. Immune responses in the intestine are mainly orchestrated by intestinal epithelial cells (IECs), dendritic cells (DCs), and T cells. Therefore, in this study we used a coculture system to investigate the effects of a soy protein and whey hydrolysate on cytokine production of human IECs, immature DCs and T cells. Both the soy and whey hydrolysate induced cytokine production (IL-12, IL-8, TNFα, IL-10, IL-1RA, IL-6, MCP-1, MIP-1α, RANTES, and TSLP) when in direct contact with DCs. In IECs soy hydrolysate only induced production of IL-8 and MCP-1. Furthermore, after coculturing IECs and DCs, the hydrolysates did not change cytokine production. T cell cytokine production was also not affected. Understanding the attenuation of the effects of these hydrolysates in intestinal immune cells contributes to a better insight in the effects of hydrolysates.

Introduction

Breastfeeding is univocally accepted as the healthiest feeding option for newborns and exclusive breastfeeding is recommended until 6 months of age [1,2]. In those situations where breastfeeding is not possible, infant formula is the only approved alternative for mother's milk [3]. Infant formulas contain proteins that are often derived from cow's milk or soy [4]. In some cases the child does not tolerate these proteins and develops an allergy against the soy or cow's milk proteins [5,6].

Hydrolyzed cow's milk and soy proteins are being used in infant formulas for treatment of infants suffering from cow's milk protein or soy protein allergy [7]. Hydrolysates are also used in infant formulas to prevent cow's milk allergy in children at risk of allergy [8]. Up to now the hypoallergenic properties of hydrolysates were assumed to be accomplished by destruction of allergenic epitopes during the hydrolyzation process [9], but recent research indicates that the peptides formed during hydrolysis also possess immunomodulatory properties [10,11]. In this way, hydrolysates could actively prevent or decrease allergic reactions by modulating intestinal immune cells [12,13]. However, the mechanisms involved in these allergy attenuating effects of hydrolysates have not been fully elucidated.

The initial immune response against a food component is mainly orchestrated by intestinal epithelial cells (IECs), dendritic cells (DCs) and T cells [14]. Especially intestinal DCs have been found to determine whether an immune response against food antigens does or does not occur [14]. The DC phenotype can be influenced by many factors, including neighboring cells [15,16] that might be modulated by food-molecules [17]. We recently demonstrated for example that dietary fibers had minor to no direct effects on DCs in monoculture *in vitro* [18]. This changed when DCs were cocultured with IECs and then incubated with dietary fibers. In this situation, DCs showed immune regulatory effects [18]. We hypothesize that similar processes may also occur with hydrolysates.

Therefore, in the present study we investigated the effects of a soy protein and a whey hydrolysate on cytokine production of human IECs, DCs, and T cells. We did this in a stepwise fashion by first studying effects of the different hydrolysates on DCs or IECs in monoculture and subsequently in a coculture of DCs and IECs. Finally, we tested the effects of DC conditioned medium on T cell polarization.

Materials and methods

Characterization of hydrolysates tested

Soy and whey protein hydrolysates were kindly provided by FrieslandCampina (Amersfoort, the Netherlands). Soy isolate and whey protein concentrate were hydrolyzed by two-step digestions to produce the hydrolysate. The peptide composition of the hydrolysates was characterized with a RP-UHPLC method. Separations were performed on a Hypersil GOLD C18 analytical column. Elution was performed with a flow rate of 0.8 mL/min. The gradient elution was carried out with a mixture of 0.1% trifluoroacetic acid (TFA) and 1% acetonitrile (ACN) in H_2O and 0.1% TFA and 90% ACN in H_2O. All samples were tested for endotoxins by using the Limulus amebocyte lysate assay (LAL) according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, US). Endotoxin concentrations in all samples had no significant effects on the cells applied.

Culturing Caco-2 cells and generating an IEC monolayer

Human epithelial colorectal adenocarcinoma Caco-2 cells were cultured in DMEM (Gibco, Life Technologies, Bleiswijk, the Netherlands) medium supplemented with 10% decomplemented fetal calf serum (FCS) (Hyclone, ThermoScientific, Breda, the Netherlands), 1% nonessential amino acids (Gibco, Life Technologies, Bleiswijk, the Netherlands), and 50 µg/mL Penicillin-Streptomycin Solution (Sigma Aldrich, Zwijndrecht, The Netherlands). When grown until ~80% confluency, cells were detached by trypsinization and seeded on top of a filter of a 24 wells transwell insert (polycarbonate membranes, 3 µm pores; Corning, NY, USA) at a concentration of 100.000 cells/well (in 200 µL). The lower chambers were filled with 1 mL of medium. Cells were maintained in the transwell inserts for 14-21 days in order to form a confluent monolayer. Medium was changed every other day. Trans-epithelial resistance was measured using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Inc.). Caco-2 cells were used for experiments when the resistance reached 400 $\Omega.cm^2$.

DCs and T cells

Autologous DCs and T cells were purchased from MatTek Corporation (Ashland, MA, USA). DCs were generated from umbilical cord blood $CD34^+$ progenitor cells (hematopoeietic stem cells). The T cells were isolated from umbilical cord blood by negative selection with Dynabeads; T cell characteristics were confirmed by FACS. 97-99% of the cells were $CD3^+$. Both DCs and T cells were cultured according to the manufacturer's instructions.

Direct stimulation of DCs and IECs

In this study, we used an *in vitro* platform to test the effect of hydrolysates on activation of different immune cells involved in intestinal immune reactions against dietary antigens as described by Bermudez-Brito et al [18]. The first step was to investigate the direct effects of hydrolysates on human DCs (figure 1A).

Freshly thawed DCs were seeded in each well of a 96 wells plate ($6x10^4$ in 200 µL). Cells were cultured for 24 hours before starting the experiment as described in the manufacturer's instructions. Then, cells were exposed to 2 mg/mL hydrolysate for 24 hours (37 °C, 5% CO_2) and supernatant was collected and stored at -80 °C for cytokine measurements (n=5).

The next step was to test the direct effects of hydrolysates on human IECs (figure 1B). To this end IECs cultured on the membrane of transwell inserts were incubated with the hydrolysates (2 mg/mL) for 24 hours, after which the basolateral supernatant was collected and stored at -80 °C for cytokine measurements (n=5) (figure 1B).

Stimulation of cocultured DCs and IECs

As the interplay and crosstalk between different intestinal cell types may be crucial for the final response [16], we also tested the effects of the whey and soy hydrolysates in cocultures of IECs and DCs (figure 1C), using a transwell coculture model mimicking the physiological situation in the intestine [18].

To study this, first DCs were thawed and seeded in 24 wells plates ($3x10^5$ cells/well) for 24 hours for the DC layer in the basolateral compartment (figure 1C). When starting the experiment the next day, a new batch of DCs were thawed and seeded at the basolateral side of an IEC monolayer

in the transwell insert membrane. Therefore, the transwell inserts were turned upside down, and $5x10^4$ DCs were seeded on the transwell membrane in 80 µL of medium. Transwell inserts were incubated upside down at 37 °C for 4 hours to allow the DCs to attach. After incubation, the medium on the membrane was removed, and the membrane was washed 2 times with PBS. Then, transwell inserts were placed in the 24 wells plate containing the precultured DCs. The hydrolysates were added to the apical chamber. After 24 hours incubation with the hydrolysates, the basolateral supernatant was collected and stored for cytokine measurements (*n*=5).

Stimulation of T cells

The last step was to determine the effect of soluble factors produced by hydrolysate stimulated DCs or by the cocultured DCs and IECs on naïve T cell activation and polarization as described before by our group [18]. To this end, T cells were seeded in a 96 wells plate at a concentration of $4x10^4$ cells/well (in 200 µL), and precultured for 24 hours. Then, 40 µL conditioned medium of hydrolysate stimulated cocultured IECs and DCs (figure 1D) or conditioned medium from DCs alone was added (n=5) (figure 1E). Unstimulated coculture medium or unstimulated DC medium was used as a control. T cells were incubated for 24 hours, and supernatant was stored for cytokine measurements.

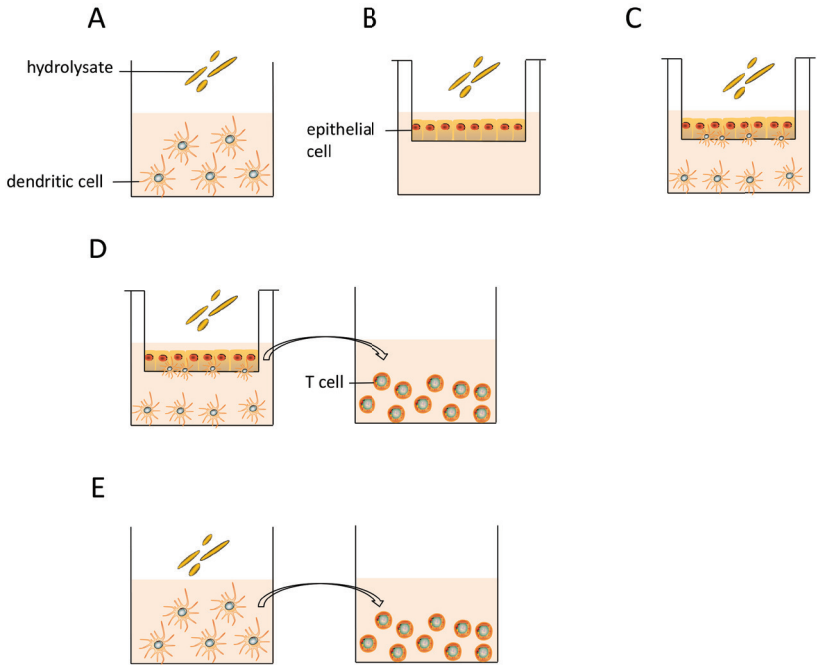


Figure 1. Overview of cell stimulations. *First, DCs (1A) and IECs (1B) alone were stimulated with soy and whey hydrolysates. Then, DCs and IECs were stimulated with hydrolysates when cultured together (1C). To do this, IECs were grown on a transwell membrane, while DCs were present both on the basolateral side of the transwell membrane and on the bottom of the basolateral compartment. To test the effects of soluble factors derived from hydrolysate stimulated cocultured IECs and DCs (1D) or from stimulated DCs alone, T cells were stimulated with the supernatant of cocultured IECs and DCs or DCs alone.*

Assessment of cytokine expression

The levels of IL-1β, IL-1RA, IL-10, IL-12, IL-6, IL-8, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, TNFα, and TSLP in the IEC and DC supernatant were measured using a custom made ProcartaPlex® multiplex immunoassays (Affymetrix, CA, USA). Another multiplex immunoassay was used to quantify the levels of IL-10, IL-2, IL-4, IL-5, and TNFα in the T cell supernatant. Both immunoassays were performed according to the manufacturer's protocol. Briefly, cytokine standards were resuspended, and serial dilutions were prepared. Antibody magnetic bead mix was added to the plate. After washing, standards and samples were added (50 µL/well), the plate was sealed, and incubated while shaking (30 min at room temperature (RT), overnight at 4 °C, and again 30 min at RT). After washing the plate twice, detection antibodies were added (25 µL/well) and the plate was incubated for 30 min at RT on a plate shaker. After incubation, the plate was washed twice and 50 µL/well streptavidin-phycoerythrin was added. Again, the plate was incubated at RT for 30 min while shaking. To prepare the plate for analysis, the plate was washed, and 120 µL/well of reading buffer was added. After shaking the plate for 5 min at RT fluorescence was measured using a Luminex 100 System. The data obtained were analyzed using StarStation software.

Statistical analysis

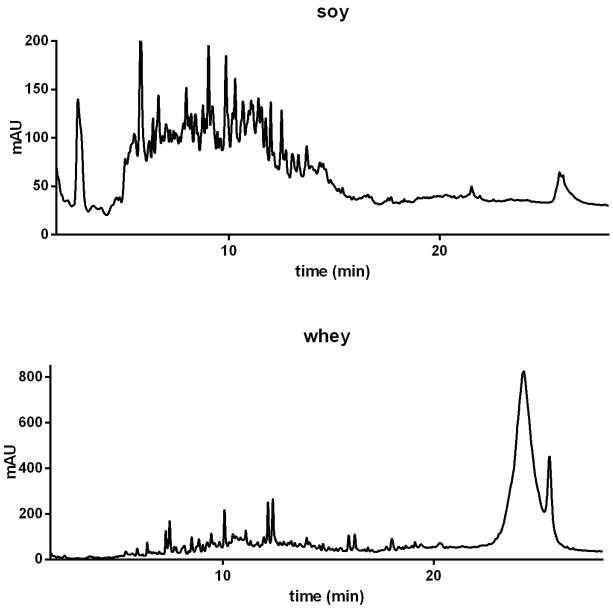
Statistical analysis was performed using Graphpad Prism 6. Normal distribution of the data was tested using the Kolmogorov-Smirnov test. All data were normally distributed. Values are expressed as mean ± standard deviation (SD). T-tests were used to show individual differences. A *p*-value of <0.05 was considered to indicate a significant difference.

Results

Characteristics of soy and whey hydrolysates

The soy and whey protein hydrolysates were obtained by a two-step hydrolysis of soy isolate and whey protein concentrate, respectively. Peptide patterns (reversed phased chromatography) and molecular weight distributions are shown in figure 2. Both hydrolysates contain mainly peptides, but also still contain a fraction of bigger (>10.000 Da), possibly intact, proteins. The soy hydrolysate tested was hydrolyzed more extensively compared to the whey hydrolysate, and therefore it contains a bigger fraction of small peptides (<500 Da).

A



B

samples	Molecular weight distribution (%)					
	>10,000 Da	10,000 - 5,000 Da	5,000 - 2,000 Da	2,000 - 1,000 Da	1,000 - 500 Da	< 500 Da
soy	4	5	8	10	16	57
whey	21	8	11	11	10	39

Figure 2. Characterization of the soy and whey hydrolysate. *The peptide composition of the hydrolysates was characterized with a RP-UHPLC method. Peptide patterns are shown in figure 2A, and a molecular weight distribution was show in 2B. Both hydrolysates contain mainly peptides, but also still contain a fraction of bigger (>10.000 Da) proteins. The soy hydrolysate tested was hydrolyzed more extensively compared to the whey hydrolysate.*

Stimulation with soy and whey hydrolysates induced strong cytokine production in DCs.

The effect of direct stimulation with the soy and whey hydrolysates on DCs was investigated. To this end, DCs were stimulated with the hydrolysates for 24 hours, and cytokines were measured in the medium using luminex (situation A in figure 1).

Both soy and whey hydrolysates significantly increased the production of IL-12, IL-8, TNFα, IL-10, IL-1RA, IL-6, MCP-1, MIP-1α, RANTES, and TSLP in DCs (all $p<0.05$) compared to unstimulated DCs (figure 3). The proinflammatory cytokine IL-1β was only significantly increased compared to the control after soy hydrolysate administration ($p<0.05$), but not after adding the whey hydrolysate.

When comparing the effects of the soy and whey hydrolysate, we found that TSLP, IL-1β, IL-12, IL-6, MCP-1, MIP-1α, RANTES, and TNFα (all $p<0.05$) were significantly higher after soy hydrolysate stimulation compared to whey hydrolysate stimulation. For IL-8, IL-10, and IL-1RA, no differences between soy and whey stimulated DCs were observed.

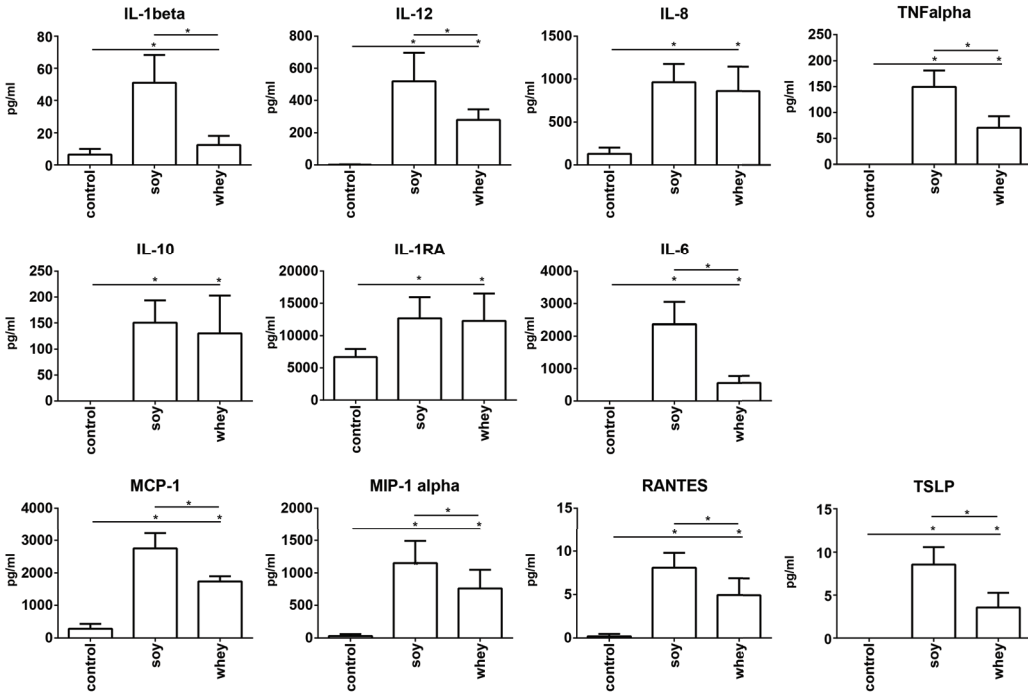


*Figure 3. Cytokine production of soy and whey hydrolysate stimulated DCs. Soy hydrolysates induced increased cytokine production of all measured cytokines and chemokines measured in the supernatant after stimulation for 24 hours. Whey hydrolysates also stimulated cytokine production for most cytokine, except for IL-1β. For a range of cytokines, the production after whey hydrolysate stimulation was less compared to stimulation with soy hydrolysate. Significant differences compared to the negative control were determined by using t-tests and indicated by *.*

Stimulation with soy hydrolysate induced IL-8 and MCP-1 in IECs.

In the intestine, the epithelial cells are the first cells to encounter dietary molecules. Therefore, we assessed the effects of soy and whey hydrolysates on cytokine production by IECs. To this end, a confluent layer of IECs was stimulated with the hydrolysates for 24 hours, and cytokines were measured in the medium using luminex (situation B in figure 1).

Only IL-8 and MCP-1 were detected in the medium of unstimulated and hydrolysate stimulated IECs (figure 4). The levels of the other cytokines did not reach detectable threshold levels of luminex (data not shown). Both IL-8 and MCP-1 were significantly increased in cells stimulated with soy hydrolysate compared to unstimulated controls ($p<0.05$), but not in cells stimulated with whey hydrolysate.

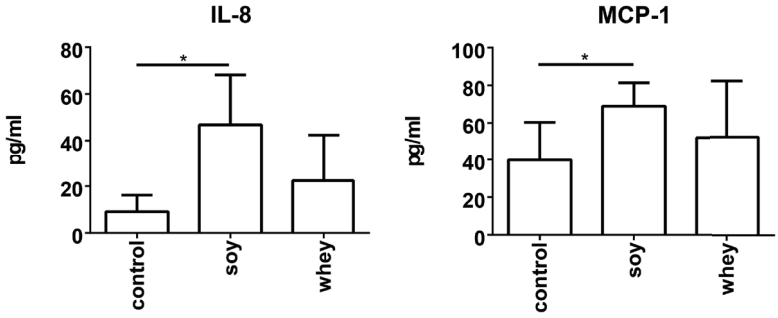


*Figure 4. Cytokine production of soy and whey hydrolysate stimulated IECs. Stimulation of IECs with soy hydrolysate for 24 hours induced increased production of IL-8 and MCP-1. This effect was not observed when cells were incubated with whey hydrolysates. Significant differences compared to the negative control were determined by using t-tests and indicated by *.*

Modulation of cytokine production by cocultured IECs and DCs

To assess the crosstalk between IECs and DCs, the next step was to investigate the cytokine response of cocultured IECs and DCs. The IEC-DC coculture was apically stimulated with hydrolysates, and after 24 hours the cytokine production by IECs and DCs was measured in the basolateral medium (situation C in figure 1).

From all cytokines measured, only IL-1β, IL-12, IL-8, IL-1RA, MCP-1, MIP-1α, and RANTES were detected in the basolateral medium obtained from the cocultured IECs and DCs (figure 5). However, for none of these cytokines the production was increased after stimulation with either the soy or the whey hydrolysate compared to the unstimulated control cells.

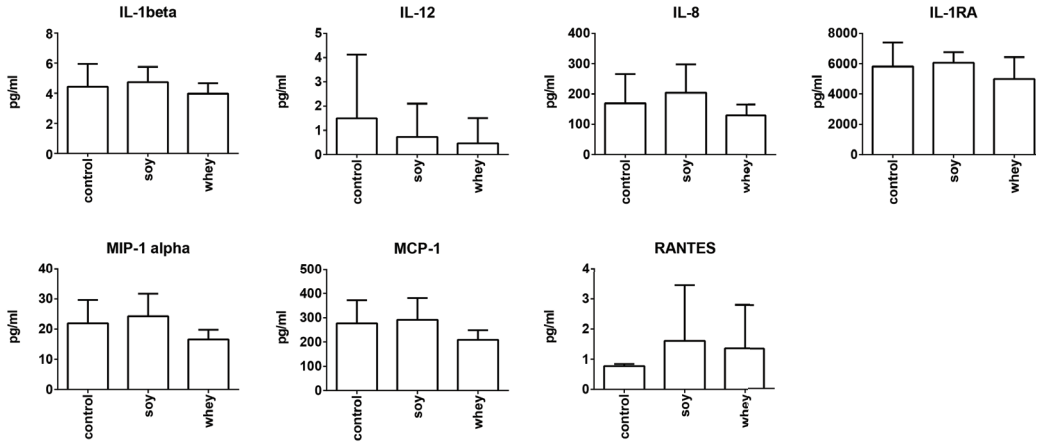


*Figure 5. Cytokine production of cocultured IECs and DCs after stimulation with soy and whey hydrolysates. When cocultured IECs and DCs were stimulated with soy or whey hydrolysate for 24 hours, the cytokines IL-1β, IL-12, IL-8, IL-1RA, MIP-1α, MCP-1 and RANTES were detected in the basolateral medium. However, no differences in cytokine production were detected between soy and whey hydrolysate stimulated cells and unstimulated cells. Significant differences compared to the negative control were determined by using t-tests and indicated by *.*

Soluble factors of hydrolysate stimulated IEC-DC cocultures did not induce T cell polarization

T cells play an important role in the immune reaction against food antigens in the intestine. The question arose whether the cytokine production by DCs (and IECs) was able to affect T cell cytokine production as reported before for dietary fibers [18]. Therefore, it was first assessed whether soluble factors in the medium of cocultured IECs and DCs, which were stimulated with hydrolysates, affected T cell differentiation (situation D in figure 1). After 24 hours of stimulation, Treg cytokine IL-10, Th1 cytokine IL-2 and Th2 cytokines IL-4 and IL- 5 were detected in the medium (figure 6). No differences in production of these cytokines were observed between groups, indicating no T cell differentiation was induced after hydrolysate stimulation.

Next, we studied whether the cytokines produced by monocultures of DCs after soy or whey hydrolysate stimulation affected T cell polarization (situation E in figure 1). Here, also IL-10, IL-2, IL-4, IL-5 and TNFα were detected in the medium (figure 7). When comparing the production of these cytokines between the groups, no significant differences between the groups were detected (figure 7).

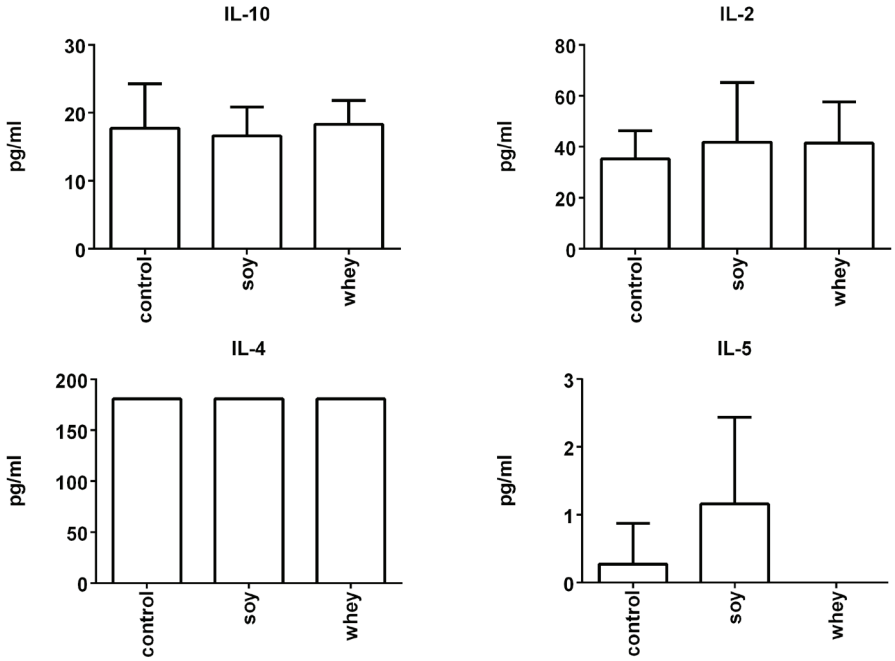


*Figure 6. Cytokine production of T cells after stimulation with soluble factors of cocultured IECs and DCs which had been stimulated with soy and whey hydrolysate. When T cells were stimulated with conditioned medium of cocultured IECs and DCs which had been stimulated with soy or whey hydrolysate for 24 hours, the cytokines IL-10, IL-2, IL-4, and IL-5 were detected. However, no differences in cytokine production were detected between soy and whey hydrolysate stimulated cells and unstimulated T cells. Significant differences compared to the negative control were determined by using T tests and indicated by *.*

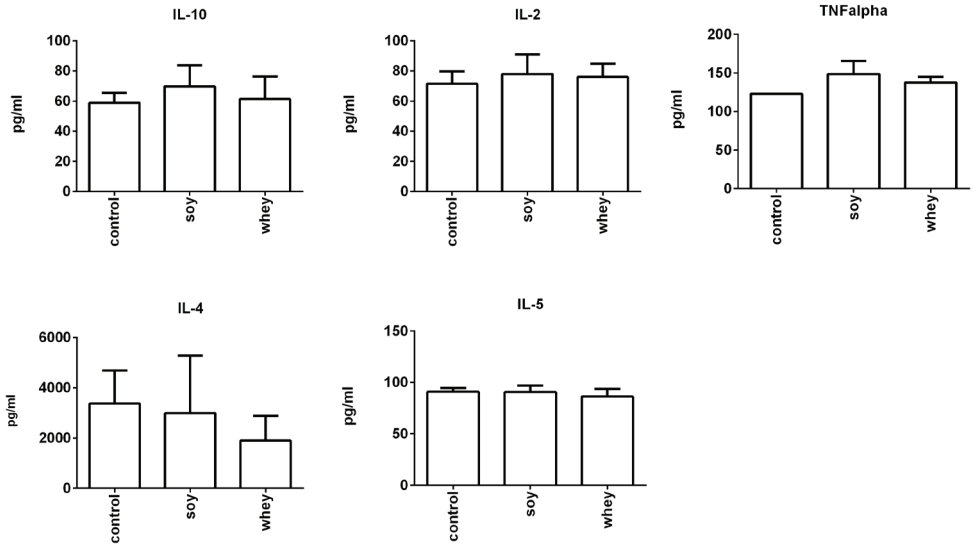


Figure 7. Cytokine production of T cells after stimulation with soluble factors of DCs which had been stimulated with soy and whey hydrolysate. *When T cells were stimulated with of DCs which had been stimulated with soy or whey hydrolysate for 24 hours, the cytokines IL-10, IL-2, IL-4, IL-5 and TNFα were detected. However, no differences in cytokine production were detected between soy and whey hydrolysate stimulated cells and unstimulated T cells. Significant differences compared to the negative control were determined by using t-tests and indicated by *.*

Discussion

Nowadays, hydrolyzed proteins in hypoallergenic infant formulas are recognized to possess a broad range of immunomodulatory effects [13,19,20]. By gaining more insight into these properties, specific hydrolysates may serve to prevent or manage unwanted immune reactions in infants. They may e.g. reduce allergenic symptoms in allergy prone infants, or improve immune maturation in infants in general. In this study, the effects of whey and soy hydrolysates on cytokine production of IECs, DCs, and T cells were investigated as possible mechanism by which these hydrolysates may modulate immunity. Both the whey and soy hydrolysate induced strong activation when in direct contact with DCs, as illustrated by increased production of IL-12, IL-8, TNFα, IL-10, IL-1RA, IL-6, MCP-1, MIP-1α, RANTES, and TSLP. In contrast, both hydrolysates had limited effects on cytokine production of IECs. Only the soy hydrolysate induced IL-8 and MCP-1 in IECs.

Despite the lack of effect on IEC derived cytokines the hydrolysates had profound effects on DCs. Since hydrolysates have been shown to induce TLR activation [21], it is plausible that the effects of hydrolysates on DCs are induced via activation of TLRs which are less present on IECs [22,23]. These direct effects on DCs may be crucial for the induction of the subsequent immune response [24]. The observed increase of the cytokines IL-6, IL-12, and IL-10 and chemokines IL-8, MIP-1α, and MCP-1 indicate maturation and activation of the stimulated DCs [25,26], which triggers more pronounced Th17 and Th1 responses [27,28]. Together with an increased IL-12 production by DCs [29], these effects are thought to contribute to the maturation of the Th2

prone, neonatal immune system towards development of Th1 responses and a therefore are decreased risk of infections [29]. Increased amounts of specific chemokines or their receptors, like RANTES/CCR5 [30], also protect against infection [31]. IL-6 and IL-10 produced by DCs leads to increased class-switching to IgA [32,33], which results in a faster clearance of pathogens or antigens [34]. However, since direct interaction of hydrolysates and TLRs is needed, mainly DCs extruding into the lumen of the intestine will be able to induce these effects.

As most DCs are present in the lamina propria, which is separated from the lumen of the intestine by the epithelial barrier, we also investigated the effects of the whey and soy hydrolysates in a coculture system in which IECs form a confluent layer on top of the DCs. In contrast to the direct effect of hydrolysates on DCs, we did not observe hydrolysate induced cytokine production in this coculture setup (figure 5). The lack of DC-stimulation by hydrolysates in the coculture setup was probably not due to regulatory cytokine production of the IECs, since none of these regulatory cytokines were observed in the coculture medium. Furthermore, stimulating DCs with conditioned medium from hydrolysate stimulated IECs confirmed that soluble factors from IECs did not affect DC cytokine production (data not shown). In the cocultures, IECs thus seem to prevent the DCs from reacting by physically protecting the DCs from contact with the hydrolysates. Crosstalk between the IECs and DCs upon hydrolysate exposure might occur, however, since IL-8 and MCP-1 are produced in monocultures of IECs upon stimulations with soy hydrolysate, but not in cocultures of IECs and DCs.

Our current data suggest that effects of soy and whey hydrolysates on immune barrier cells are very different from that of non-digestible fibers as we reported before and which was studied in the same cell system [18]. The currently tested hydrolysates have strong direct effects on DCs, while non-digestible carbohydrates had only minor direct effects on DCs [18]. In contrast, the dietary fibers were potent activators of IECs, which were key in inducing enhanced DC and T cell responses due to soluble factors secreted by IECs. This IEC conditioning effect was not observed for whey and soy hydrolysates. This emphasized the notion that effects of food components on the consumers immune system is complex but allows for tailored intervention by selecting specific molecules with confirmed effects for managing specific disorders.

Since it has been shown with dietary fibers that soluble factors derived from fiber stimulated DC and IEC cocultures are able to skew T cell differentiation [18], the last step in our experiment was to determine whether hydrolysates could have a similar effect on T cell polarization. However, hydrolysate stimulated DC and IEC coculture conditioned medium did not affect T cell differentiation (figure 6). Also, since activation of DCs are known to induce T helper 1 cell differentiation, we tested the effect of directly stimulated DC medium on T cell differentiation. But again, no altered differentiation of T cells was detected *in vitro*, as evidenced by absence of cytokine enhancements such as reported for dietary fibers (figure 7). This indicates again that effects of hydrolysates are very different from that of dietary fibers and mainly influence DCs [35].

In summary, both the soy and the whey hydrolysate have been shown to induce strong cytokine production in DCs. The presence of a layer of IECs controls this strong response in DCs. T cells were not affected by soluble factors of IECs and DCs. The present knowledge leads to a better understanding of the immune regulating effects of hydrolysates and can ultimately be used to improve hypoallergenic infant formulas and infant formulas in general.

References

1 Eidelman, A.I., Schanler, R.J., Johnston, M., Landers, S. Breastfeeding and the use of human milk. Pediatrics.2012 Mar;129(3):e827-41.doi: 10.1542/peds.2011-3552.Epub 2012 Feb 27.

2 Kramer, M.S., Kakuma, R. Optimal duration of exclusive breastfeeding. Cochrane Database Syst.Rev. 2012,CD003517.

3 Pina-Perez, M.C., Martinez, A., Rodrigo, D. New Advances in Infant Feeding: New Products and Novel Technologies. Recent Pat Food Nutr Agric.2017 Mar 28.doi: 10.2174/2212798409666170328145150.

4 Andres, A., Cleves, M.A., Bellando, J.B., Pivik, R.T., et al. Developmental Status of 1- Year-Old Infants Fed Breast Milk, Cow's Milk Formula, or Soy Formula. Pediatrics. 2012,129,1134-1140.

5 Patel, B.Y., Volcheck, G.W. Food Allergy: Common Causes, Diagnosis, and Treatment. Mayo Clin.Proc. 2015,90,1411-1419.

6 Cordle, C. Soy protein allergy: Incidence and relative severity. J.Nutr. 2004,134,1213S- 1219S.

7 von Berg, A., Koletzko, S., Grubl, A., Filipiak-Pittroff, B., et al. The effect of hydrolyzed cow's milk formula for allergy prevention in the first year of life: The German Infant Nutritional Intervention Study, a randomized double-blind trial. J.Allergy Clin.Immunol. 2003,111,533-540.

8 Terracciano, L., Isoardi, P., Arrigoni, S., Zoja, A., Martelli, A. Use of hydrolysates in the treatment of cow's milk allergy. Ann.Allergy Asthma Immunol. 2002,89,86-90.

9 Bu, G., Luo, Y., Chen, F., Liu, K., Zhu, T. Milk processing as a tool to reduce cow's milk allergenicity: a mini-review. Dairy Sci.Technol. 2013,93,211-223.

10 Gauthier, S.F., Pouliot, Y., Saint-Sauveur, D. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. Int.Dairy J. 2006,16,1315-1323.

11 Santiago-Lopez, L., Hernandez-Mendoza, A., Vallejo-Cordoba, B., Mata-Haro, V., Gonzalez-Cordova, A.F. Food-derived immunomodulatory peptides. J Sci Food Agric.2016 Mar 4.doi: 10.1002/jsfa.7697.

12 Lozano-Ojalvo, D., Lopez-Fandino, R., Wu, C.S., Li, Y.R., et al. Immunomodulating peptides for food allergy prevention and treatment. Crit Rev Food Sci Nutr.2017 Jan 19:0.doi: 10.1080/10408398.2016.1275519.

13 Kiewiet, M.B.G., Gros, M., van Neerven, R.J.J., Faas, M.M., de Vos, P. Immunomodulating properties of protein hydrolysates for application in cow's milk allergy. Pediatric Allergy and Immunology. 2015,26,206-217.

14 Ruiter, B., Shreffler, W.G. The role of dendritic cells in food allergy. J.Allergy Clin.Immunol. 2012,129,921-928.

15 Iliev, I.D., Spadoni, I., Mileti, E., Matteoli, G., et al. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut. 2009,58,1481-1489.

16 Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., et al. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat.Immunol. 2005,6,507-514.

17 de Kivit, S., Kostadinova, A.I., Kerperien, J., Morgan, M.E., et al. Dietary, nondigestible oligosaccharides and Bifidobacterium breve M-16V suppress allergic inflammation in intestine via targeting dendritic cell maturation. J Leukoc Biol.2017 May 11.pii: jlb.3A0516- 236R.doi: 10.1189/jlb.3A0516-236R.

18 Bermudez-Brito, M., Sahasrabudhe, N.M., Rosch, C., et al. The impact of dietary fibers on dendritic cell responses in vitro is dependent on the differential effects of the fibers on intestinal epithelial cells. Mol Nutr Food Res.2015 Apr;59(4):698-710.doi: 10.1002/mnfr.201400811.Epub 2015 Mar 2.

19 Gauthier, S.F., Pouliot, Y., Saint-Sauveur, D. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. Int.Dairy J. 2006,16,1315-1323.

20 Gill, H., Doull, F., Rutherfurd, K., Cross, M. Immunoregulatory peptides in bovine milk. Br.J.Nutr. 2000,84,S111-S117.

21 Kiewiet, M.B.G., Dekkers, R., Gros, M., van Neerven, R.J.J., et al. Toll-like receptor mediated activation is possibly involved in immunoregulating properties of cow's milk hydrolysates. PLoS One.2017 Jun 8;12(6):e0178191.doi: 10.1371/journal.pone.0178191.eCollection 2017.

22 Melmed, G., Thomas, L., Lee, N., Tesfay, S., et al. Human intestinal epithelial cells are broadly unresponsive to toll-like receptor 2-dependent bacterial ligands: Implications for host- microbial interactions in the gut. Journal of Immunology. 2003,170,1406-1415.
23 Suzuki, M., Hisamatsu, T., Podolsky, D. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular toll-like receptor 4-MD-2 complex. Infect. Immun. 2003,71,3503-3511.
24 Hemmi, H., Akira, S. TLR signalling and the function of dendritic cells. Chem Immunol Allergy. 2005;86:120-35.
25 Kranzer, K., Eckhardt, A., Aigner, M., Knoll, G., et al. Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. Infect.Immun. 2004,72,4416- 4423.
26 Ahonen, C., Gibson, S., Smith, R., Pederson, L., et al. Dendritic cell maturation and subsequent enhanced T-cell stimulation induced with the novel synthetic immune response modifier R-848. Cell.Immunol. 1999,197,62-72.
27 Denning, T.L., Wang, Y., Patel, S.R., Williams, I.R., Pulendran, B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat.Immunol. 2007,8,1086-1094.
28 Mohamadzadeh, M., Olson, S., Kalina, W., Ruthel, G., et al. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. Proc.Natl.Acad.Sci.U.S.A. 2005,102,2880-2885.
29 Zaghouani, H., Hoeman, C.M., Adkins, B. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. Trends Immunol. 2009,30,585-591.
30 Santiago, H., Oliveira, C., Santiago, L., Ferraz, F., et al. Involvement of the chemokine RANTES (CCL5) in resistance to experimental infection with Leishmania major. Infect.Immun. 2004,72,4918-4923.
31 Aliberti, J., Sousa, C., Schito, M., Hieny, S., et al. CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha(+) dendritic cells. Nat.Immunol. 2000,1,83-87.
32 Mora, J.R., Iwata, M., Eksteen, B., Song, S., et al. Generation of gut-homing IgA- secreting B cells by intestinal dendritic cells. Science. 2006,314,1157-1160.
33 He, B., Xu, W., Santini, P.A., Polydorides, A.D., et al. Intestinal Bacteria Trigger T Cell- Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. Immunity. 2007,26,812-826.
34 Mantis, N.J., Rol, N., Corthesy, B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunology. 2011,4,603-611.
35 Kapsenberg, M. Dendritic-cell control of pathogen-driven T-cell polarization. Nature Reviews Immunology. 2003,3,984-993.